THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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Application for Research Grant

(Use extra pages as needed)

Date: Feb. 4, 1974

1. Principal Investigator (give title and degrees):

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(FEB 7 1974)

2. Institution & address:

Saint Lukes Hospital, 11311 Shaker Blvd., Cleveland, Ohio 44104

3. Department(s) where research will be done or collaboration provided:

Pathology Research

4. Short title of study:

Studies of Carcinogenesis in Organ Culture of Trachea and Bronchi

5. Proposed starting date: 7-1-74

6. Estimated time to complete: two years

7. Brief description of specific research aims: The specific overall objectives of the work proposed in this research application are to develop an optimal system of organ culture of hamster trachea, bronchi and bronchioles and of human trachea, bronchi and bronchioles which will permit survival of the cells and tissues in as near an optimal state as possible (compared to precultured tissue) for a period of time of sufficient duration to develop malignant neoplasms following the introduction of single pure chemical carcinogens within the culture medium; to evaluate methods of determining viability within the explants of trachea and bronchi; and to determine the true malignant character of any tumor which may be induced in the in vitro system by re-inoculation or transplantation into a living animal, and observation and study of the implant for growth and metastases. Studies in isolated systems, such as those proposed, allow for more specific and direct evaluation of the true time course, the target cell of origin, and the opportunity for biochemical evaluation of the earliest lesion associated with premalignant and overtly malignant tissue. Ultrastructural studies may also provide an indication of the cell type, the organelles affected and the temporal course of the effects of several carcinogens on the cell organelles.

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8. Brief statement of working hypothesis:

The purpose of the present research proposal is to develop an optimal system of organ culture for maintenance of trachea, bronchi and bronchioles (lung tissue) for prolonged periods and to develop methods of inducing carcinogenesis in the cultured trachea and bronchiolar tissue by exposure to carcinogenic chemicals in culture. This work is based upon the premise that respiratory tissue in organ culture will respond more specifically to stimuli induced by carcinogenic chemicals toward the development of malignant transformation. Relatively isolated systems eliminate hormonal influences, immunological effects and other systemic factors including perhaps viral infections which can influence malignant transformation in vivo. Attempts will be made to produce a system in which the tissue explants will survive for longer periods and may therefore have greater opportunity to develop malignant transformation under the influence of chemical carcinogenic stimuli. Both human and animal models will be studied in order to compare their effects and transfer observations when they are useful. Realistic and critical means of evaluation of suspected neoplastic transformations will be utilized to evaluate this change. Confirmation of the neoplastic transformation will include histological, ultrastructural, biochemical and transplantability criteria. If these objectives are accomplished, a standardized and reliable method of observing the early cellular and organoid changes in the development of a malignant neoplasm will be provided, and a manipulable and reproducible model for inducing and studying the initial and progressive alterations in the afflicted cell or cells will be available for both biochemical and morphologic (including ultrastructural) study. A by-product of this research will be the development of standardized and uniform methods for testing the effect of various chemicals for carcinogenic potential on respiratory tract epithelium.

9. Details of experimental design and procedures (append extra pages as necessary):

A. Background:

The technique of organ culture offers a unique compromise in the study of chemical carcinogenesis. It eliminates the many confusing limitations in working with whole animals and yet preserves the organoid community of cells and tissues which together give any organ its unique structural and functional identity. Organ culture of lung was first practiced by Fell (1) on embryonic organ fragments and femurs. In 1959 Trowell (2) systematically evaluated the maintenance and growth of mature organs in an artificial culture system. Trowell demonstrated that epithelial cells did better when massed with minimal stroma and that mitoses could be observed in epithelium. He specifically sustained lung and noted, as was our experience, that the alveolar lumens are overgrown by enlarged alveolar epithelial cells. Lasnitzki (3) studied the effect of 3-4 benzpyrene on human fetal lung in organ culture and showed the outgrowth of bronchioles, alveoli and cartilage. The carcinogen produced bronchiolar hyperplasia and abnormal mitoses but inhibition of connective tissue growth. Similar changes were observed by this same author using four fractions of cigarette smoke condensates, but different types of epithelial cell hyperplasia and metaplasia were observed with the various types of condensates. Cailleau, Crocker and Wood (4) reported the long term culture of human bronchial mucosa and neoplasm in 1959. Ciliary activity was observed for as long as 135 days and mucous material in glands up to 21 days. Tumor tissue did not survive for long periods. Sorokin (5) described the development in organ culture of mammalian fetal lung. Most components of lung developed but vascular tree growth was restricted. He noted nearly full potential for lung formation and differentiation. The effects of "epithelial growth factor" (EGF) on organs in organ culture were described by Jones (6). His studies suggested stimulation of lung growth by EGF, but careful control studies were not performed. Simnett and Hepplestom (7) reported significant differences in mitotic index of lung alveolar tissue in organ culture relating to sex, strain and age differences. After three days of culture, these differences were reduced; these authors suggested that sex and age cultures may be hormonally induced but strain differences were persistent and inherent. These same authors (8) studied the mitotic incidence (MI) in lung alveolar tissue of newborn, three and twelve month mice in organ culture for 22 days. They concluded that cell division of lung alveolar tissue is controlled in part by a tissue inherent mitotic inhibitor, present in adult lung but lacking in neonatal, in association with other stimulation factors possibly hormonal. Sonokin and Adelstein (9) reported the effect of 1100 rads of x-ray given 24 hours before explantation

on the development of organ cultures of lung. Ciliogenesis appeared to proceed normally from centrioles (redimentary cilia) and basal bodies still unformed after X irradiation, even though the cell proper has suffered a dose which will impair its proliferation. Davis (10) described a somewhat different technique for the maintenance of guinea pig lung in organ culture. His method consisted of a flask-like apparatus with two stopcocks. Within the chamber the tissue is supported, nourished and oxygenated. By this method the lung was maintained for 14 days; after 6-7 days the alveolar spaces became solidly filled with alveolar epithelial cells. Blood vessels (except for endothelium) and bronchioles are well maintained. Ultrastructural study confirmed the normalcy of the cellular structures. Lasnitski (11) observed that a hydrocarbon rich fraction from cigarette smoke condensate uniformly caused the growth of new bronchi, caused epithelial enlargement, stimulated mitoses, and induced bronchial epithelial hyperplasia in human fetal lung in organ culture. The effect was more widespread in younger tissue. Dirksen and Crocker (12) reported the ultrastructural alterations of respiratory epithelium produced by polycyclic aromatic hydrocarbons on suckling rat trachea in organ culture. Several potent carcinogens including 7, 12, dimethylbenz(a)-anthracene (DMBA) and benzo(a)-pyrene (BP) produced cells with little endoplasmic reticulum, many free ribosomes, abundant cytoplasmic filaments and autophagic vacuoles. DMBA produced these abnormalities at low concentrations in contrast to the other compounds. Cherry and Taylor Robinson (13) reported a method for the production of large quantities of tracheal organ cultures in roller type tubes. This has not been attempted for large scale production of lung explants. These studies indicate the complete feasibility of successful organ culture of lung tissue.

The subject of bronchopulmonary carcinogenesis has been reviewed recently and some of the facts relevant to this investigation will be considered in the following discussion (14,15,16). There is a considerable lag in progress made on cancer of the respiratory tract when compared with other organs and systems. The difficulty in experimentally inducing respiratory tumors has been overcome lately with the introduction of the Syrian golden hamster as the animal of choice by Della Porta, Kolb and Shubik (17). These authors produced bronchogenic carcinoma in hamsters by repeated intratracheal applications of a suspension of dimethylbenzanthracene in a 1% gelatin colloid. These observations were later extended to benzpyrene in Tween 60 or olive oil (18). These results could not always be reproduced and therefore an important step ahead was taken when Saffiotti, et al (19), injected intratracheally into hamsters a suspension of a mixture of benzpyrene and hematite. This method consistently produces a high number of respiratory tract tumors in vivo, with generally negligible inflammation or irritation. For successive papers, this group of investigators has carefully studied the conditions of their model (20,21). It has become evident by then that in the lung, as in skin and other organs, the vehicle or solvent used was of paramount importance in the induction of tumors. Kuschner (22), for example, has shown that large, repeated intratracheal doses of benzpyrene without carrier dust failed to produce cancer in hamsters while 3-methylcholanthrene given in the same manner was a potent respiratory carcinogen. To date no systemic study on the role of vehicles in the production of bronchogenic tumors has been reported. Such studies are included in the present proposal. As a result of their use of wire-mesh pellets impregnated with polycyclic hydrocarbon carcinogens and studies on the particle size of the carcinogen and its carrier dust, Kuschner (23) and Saffiotti (24) respectively have considered the possibility that the carcinogenetic potency of a given compound may be related, among other factors, to its retention by the bronchial tissues. Experiments in this proposal are designed to obtain information that, hopefully, will clarify the matter.

Saffiotti's group has recently demonstrated that pharmacological doses of vitamin A can inhibit considerably the induction of tumors by benzopyrene - Fe_2O_3. A similar

observation had been made in 1943 by Rosicky and Hatschek on mouse-skin tumorigenesis by benzpyrene. The biochemical mechanism of this phenomenon is difficult to evaluate; several possibilities for further studies are outlined here.

In 1958, Magee and Barnes (25) discovered the carcinogenicity of dimethylnitrosamine. N-nitroso compounds not only occur in foods and tobacco smoke, but can be found in vivo. Tracheobronchial tumors have been produced in hamsters by the administration of diethylnitrosamine by lavage, feeding or intratracheal injection (26). Dontenwill and his colleagues (27) have extensively studied this alkylating agent and have shown that it is a systemic carcinogen with positive organotropism for the liver in rats and for the respiratory tract in the hamster, regardless of the route of administration.

In vitro studies on respiratory tract carcinogenesis have met with only limited success. As Kuschner and Laskin (23) point out, "...even here convincing changes beyond the induction of striking atypical epithelial alterations have not been achieved." A vast literature is available on mechanisms of carcinogenesis in other organs (liver, skin, hematopoietic system), much of which is probably applicable to the respiratory system (see for example, references 28,29.30). However, because of its peculiar morphology, function and biochemistry, such applicability will have to be established.

B. Choice of Animal Species for Study

1. Organ culture techniques will be explored to evaluate the best combination of external gaseous environment, media, temperature or incubation and method of culture. The tissue of choice will be that of the Syrian hamster. This animal has been widely accepted as the one of choice for in vivo studies involving experimental production of lung tumors for several reasons. First, the incidence of spontaneous development of pulmonary neoplasms is exceedingly low, and second, this animal has great natural resistance to infection and is not easily subject to incidental pulmonary infections. Moreover, because of the ultimate necessity for re-transplantation of any tumors generated in organ culture into intact animals of the same species, animals of an inbred strain should be utilized. One such strain exists in this country. This strain Bio #8720, is obtainable from the Trenton Experimental Laboratory Animal Company, Bar Harbor, Maine. Study of organ cultures of trachea and lung tissue from suckling and adult (80-100 gm) hamsters of this inbred strain will be utilized. In addition to studying the trachea and bronchus, the peripheral lung tissue will be cultured. The purpose of the lung tissue culture is to evaluate the survival and integrity of the intrapulmonary bronchioles. In our experience these structures are well preserved in organ cultures and have all the advantages of remaining within their natural pulmonary environment within a lung slice, while containing all of the epithelial structures characteristic of bronchial or bronchiolar structures. For these reasons they represent an excellent source of bronchiolar tissue for study.

2. Human Bronchial and Bronchiolar Tissue

Human material from surgical and autopsy cases where spontaneous disease is minimal or absent will also be used in the organ culture studies. Because of the close proximity and intrinsic relationship of the autopsy and surgical pathology service to the investigator, material from selected cases may be available for culture within 20-30 minutes after death. This in large measure is due to the well organized pathology assistant staff which is an integral part of the autopsy room staff at St. Luke's Hospital and to the immediate supervision and control of autopsy material by the investigator. Other studies involving pediatric and neonatal tracheal material have been successfully used by collaborrating investigators at affiliated institutions for organ culture studies. This attests to the acceptibility of the human material and the ease in obtaining it immediately after death.

C. Method of Performing Organ Culture Studies

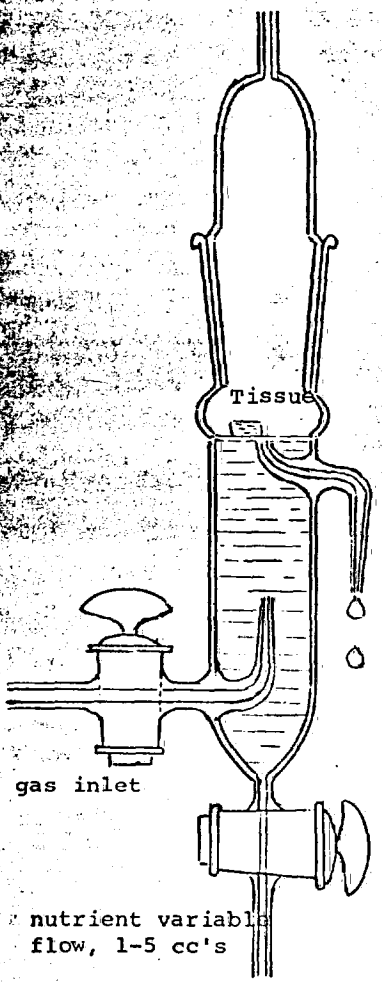
1. The primary method of organ culture to be explored will be that of Trowell. In this method, to be described in greater detail in the body

of this section, the tissue fragment or explant is supported on lens paper or rayon on a metal grid, the bottom surface of which is immersed in culture fluid.

2. Modifications of the Trowell (2) technique will also be studied. These will include the attachment of the tissue fragment to the base of a plastic petri dish which is scarified with a knife blade. Multiple fragments are placed in various separate locales on the Petri dish surface and submerged in culture fluid.

3. The Cherry Technique (13). In this method tracheal, bronchial or lung fragments are placed in roller tubes in 1-1.5ml. of culture fluid and rotated continuously. This technique will be evaluated for preservation of the tissue and maintencance of good cell integrity.

4. The Modified Davis Technique (10).



In this method, reported by Davis in 1967, the lung tracheal or bronchial tissue is maintained on a platform of rayon or wire mesh which is supported (like a fritted glass filter) on the bottom half of a glass chamber. This glass chamber is connected by two stopcocks to the nutrient medium source and to the atmospheric gas. The top portion of the chamber leads via a millipore filter to the outside atmosphere. This technique is worthy of further modification and study because it is small and readily adaptable to a slow, continuous perfusion system for nutrient and atmospheric gas supply to the fragment of tissue. This methodology may be useful in attempting to maintain viability, of the organ culture fragment for periods of over 21-28 days. It would appear to this investigator that alteration of the Davis technique to provide continuous pump-fed nutrient supply at a very slow rate, and a continuous flow system for atmospheric gas are two of the vital parameters which must be explored in attempts to prolong the viability of the cultures.

5. In addition to the above methods, the following variation in culture parameters will be attempted to produce optimal tissue survival conditions.

a. Evaluation of the Gas Tensions in the Atmosphere.

While many studies of organ culture of lung and other tissues have utilized atmospheres of 95-97% oxygen and 5-3% CO_2, recent studies have suggested that this concentration of oxygen may be injurious even to respiratory tract epithelium for long periods. In studies soon to be reported (Boat, Kleinerman, et al)(31) the principal investigator has had the opportunity to evaluate the effects of 95-100% oxygen on human tracheal tissue in organ culture. Prolonged exposure to this high oxygen atmosphere produces unequivocal injury to mucosal epithelium and glands. Preliminary studies suggest that it would be more reasonable to keep the atmospheric oxygen concentration at 50-70% O_2 and 50-30% nitrogen and 3% CO_2. (Note: 40-50% would probably to optimal in vivo, but the increased concentrations will be helpful in providing adequate oxygen tensions in the lower layers of the submerged cultures.)

b. Evaluation of the Effects of Temperature.

Recent studies have suggested that cultures of trachea and bronchi may be better maintained at 32-34°C. instead of the usual 37°C. This temperature will be evaluated.

c. Variations in culture media.

In recent years investigators have attempted to simplify the complex supplemental medias used originally in organ culture. In the past we have successfully cultured hamster lung and trachea using Trowell's T-8 media supplemented with chick embryo extract, horse serum and antibiotics. Since a sizable quantity of cultured tissue will be necessary for adequate viability and biochemical assays, it would seem economically desirable to utilize a simplified growth media. Therefore, we propose to evaluate the following medias using the Trowell, modified Trowell, roller tube and modified Davis chamber methods of culture:

1. Trowell T-8, horse serum, penicillin and streptomycin with and without 10% or less chick embryo extract, pH 7.0-7.2;

2. 199, horse serum, penicillin and streptomycin with and without 2% or less chick embryo extract, pH 7.0-7.2;

3. Basal Eagles Medium (buffered with Hepes), penicillin and streptomycin with and without 0.2% or less bovine albumin, pH 7.0-7.2. The use of the Hepes buffer will maintain the orginal pH of the media for up to 15 days using the roller tube technique for tracheal cultures, as suggested by Cherry and Taylor-Robinson.(13).

d. The Effect of Vitamin A

The role of vitamin A in maintaining the integrity of mucosal and epithelial surfaces has been classically demonstrated by the work of Fell and Mellanby (32), Laznitski (33) and Aydelotte (34). Laznitski has demonstrated the effectiveness of vitamin A in maintaining mucosal characteristics in in vitro situations. More recently Saffiotti (19) and associates have reported the inhibitory effect of vitamin A on the induction of squamous metaplasia and neoplasms in hamsters by combinations of benzpyrene and hematite administered intratracheally. The concentrations of vitamin A in the culture media will be varied in order to evaluate those doses which are optimal for preservation of epithelial integrity. Vitamin A will be utilized in final concentrations of 10-20 international units per milliliter (0.01-0.02 millimoles). Media deficient in vitamin A will contain a total absence of this material. Vitamin A will be introduced into the culture medium in the form of vitamin A alcohol (13-Cis-retinol). The systematic investigation of 30-50 I.U./ml of culture media will also be studied. This data will be useful in evaluating the effects of higher doses of vitamin A when they are used in conjunction with carcinogens introduced directly into the culture media.

6. Monitoring of media for bacterial and mycoplasma infection.

Growth media will be cultured at the time of harvest of the explant for bacterial or mycoplasma infection. Any contaminated organ culture will be immediately eliminated from further analysis.

D. New Methods to be Investigated in Conjunction of Organ Culture Approaches

1. Separation and cultivation of mucosa after detachment from cartilage and adventitial tissue.

Full thickness sections of human trachea may at times exceed 2 mm of thickness. This excessive accumulation of tissue elements may interfere with the proper nutrition of the submucosal elements, both epithelial and connective tissue. Better nutrition will be achieved when cartilagenous tissue and excessive adventitial connective tissue can be removed. Techniques to effect this separation have been successfully applied in our laboratory. The mucosa, superficial connective tissue, and glands can generally be separated from underlying cartilage. We have accomplished this in human tracheal tissue and animal tissue other than hamster where an adequate amount of submucosal tissue and glands exist.

Technique: A #30 stainless steel needle is introduced between the submucosal and cartilage and a balanced salt solution is gently injected to create a plane of cleavage. Gentle dissection is used to separate the mucosa and glands from the underlying cartilage, which will then be cultured separately by the organ culture techniques previously described.

2. Effect of macrophages with and without hematite and benzapyrene on the maintenance growth of tracheal and bronchial organ cultures.

The recent observations of Saffiotti, Cefis and Kolb (19) have suggested that the interaction of dust particles and macrophages may influence the localization of neoplasmas in the bronchial tree generated by the introduction of dust and benzapyrene into the tracheobronchial tree. It has been suggested that the carcinogen absorbed on the dust particle surface penetrates the respiratory epithelium and is phagocytized by macrophages. It is eluted from the macrophages by plasma and macrophage disintegration and diffused along the mucosal surface to impregnate the deeper mucosal layers at various sites in the bronchial tree. This hypothesis has not completely evaluated the role of the macrophage in the processing of the carcinogen into an active state nor its role in delivering carcinogens to the lower layers of the epithelium by process of active motility and insinuation within the cells of the mucosa. These possibilities can be evaluated using an organ culture system with trachea. The general outline of the study will be as follows:

Macrophages will be harvested from the lung of each animal whose lung will be subsequently cultured. If this technique appears to injure the lining of the epithelium and interferes with the proper growth of the trachea in culture the technique will be modified to harvest macrophages from the peripheral bronchi and lung without exposing the major portion of the trachea to the washout process. Autologous macrophages harvested by perfusion of lung with normal saline will then be centrifuged and resuspended in growth media with and without serum. Aliquots of macrophages will be incubated with the following materials: 1) hematite dust of 99% less than 10 micra in diameter, 94.1% less than 1 micra in various concentrations so as to produce optimal macrophage ingestion of the particulate, 2) mixture of equal weights of benzapyrene (purified) and hematite ground together in a mortar to yield a finely divided homogeneous dust containing 50% each of benzapyrene and hematite by weight, 3) a solution of benzapyrene alone dissolved in acetone and diluted with growth media to final concentration equal to that present in the particulate form, 4) macrophages exposed to an inert dust such as carbon black in sufficient concentration to produce ingestion in considerable quantities by the macrophages, and 5) macrophages alone without dust or benzapyrene exposure.

Autologous tracheal explants (the same animal from which macrophages were collected) will be exposed to concentrations of 1×10^6 macrophages containing various combinations of hematite, carbon and benzpyrene and hematite, benzpyrene and macrophages alone. The effects of single exposures will be evaluated over the life period of the organ culture. Since limited numbers of explants are available, harvest will only be performed in the preliminary studies at weekly intervals.

In another series of experiments again utilizing autologous macrophage and tracheal explants the longitudinal effects of dust alone in macrophages, benzpyrene plus dust and benzpyrene alone are studied by

utilizing harvesting at 2,4,7,14,21 and 28 days and at weekly intervals thereafter. One hamster will be used for the longitudinal evaluation of each of the variables to be studied. This will provide an adequate number of explants to allow for harvest and study of explants over longer periods of time. At least five or six hamsters will be used to evaluate each of the variables.

The ultimate goal of this study will be to analyze the role of the alveolar macrophage in the processing of carcinogen benzpyrene and its ultimate effect in inducing carcinogenesis. The role of the so-called inert dust, hematite, may also be evaluated as compared to the carbon black, since epidemiological studies reported from Great Britain in hematite miners suggest an increased prevalence of primary lung cancer in this group.

E. Morphological Evaluation

1. Periodic harvest and histological evaluation.

At predetermined intervals organ culture explants of trachea, bronchi and lung will be harvested, fixed in neutral buffered formalin or Bouin's solution and processed by conventional techniques of 4-6 micra thickness. Harvest interval in its early experimental studies will be 1,2,4,7,14,21, and 28 days and at weekly intervals thereafter for as long as cultures are viable or cultures are available for harvest. Our present technique allows for at best twelve segments of hamster trachea in the form of half rings to be avilable from each animal. These will be distributed among different culture dishes so that each dish will contain an equal number of fragments from upper, middle and lower portions of the trachea. This sampling will be utilized to avoid any local effects related to trauma or differences in trachea metabolism. A similar number of explants from peripheral lung tissue which includes medium sized bronchi and bronchioles will be cultured and harvested simultaneously. In addition to regular histology, special stains to evaluate the acid mucopolysaccharide (AMP) of the cultured tracheal cells will be performed. Alcian blue/PAS at pH 2.5 and pH 1.0 with and without sialidase digestion. In addition, Baker stain for bound phospholipids will be performed to evaluate the secreting abilities of the Clara cell in the bronchioles. The general character of the epithelium, the height and size of the cells, the relative proportion of each cell type, the mucus secreting abilities of the secretory cells and the cytologic character of each of the cell types will be evaluated by light microscopy techniques. In addition the napthylamidase (35) reaction will be performed on selected explants which will be harvested from each of the various experimental studies. The napthylamidase technique is performed on frozen sections at pH 5.5 to demonstrate lysosomal cathepsin B activity. This histochemical method is said to demonstrate cell injury and lysosomal enlargement in a stage much before changes can be demonstrated by other techniques. By means of this technique sections

incubated with Leu-2-napththylamide (LNA) at pH 5.5 in a 0.1 molar acetate buffer for 1-2 hours in the primary reaction produce 2-napthyl-amide, simultaneously coupled to diazo blue B and heated finally with $CuSO_4$ to produce a blue or violet chelated end produce. This test can provide evidence of early cell injury and act as an index of incipient cell damage.

2. 1 micron Epon embedded section

When it has been established that reasonable cell survival and integrity has been accomplished in the culture system by light microscopic analysis, additional studies utilizing 1 micron thick Epon embedded sections and light microscopic analysis will be performed. By means of this technique a relatively rapid but more detailed analysis of cell types and cell characteristics will be observed. In this analysis morphometric techniques for evaluating the relative proportion of cell types, cell size and intracellular organelles will be evaluated.

3. Ultra thin sections for electron microscopy

When necessary ultra thin sections for electron microscopy will be prepared from the tissue embedded in epon for more detailed analysis of cell membrane, ciliary characteristics, mitochondrial structure, number, size and character of liposomes, amount and distribution of rough and smooth endoplasmic reticulum and the number and size of characteristic secretory granules. This analysis will be performed on both control and carcinogen exposed cultures as a means of evaluating the optimal organ culture system to support the long growth of tracheal, bronchial and lung explants.

4. Autoradiography

Cell viability and replication will be evaluated by the application of radioautographic methods.

1. Tritiated thymidine incorporation (H^3Thymidine). The uptake of this isotope will be used as an index of DNA synthesis. Incorporation into the nuclei of viable replicating cells occurs during the "S" phase of the cell cycle. Tritiated thymidine will be introduced into the culture medium in concentrations of 0.05 uc/ml or 0.10 uc/ml (specific activity 1.9 c/mmole). This low concentration is used so as to produce no radiation injury to the cells. After a pulse label of 2-4 hours, the labelled thymidine will be removed and fresh, nonlabelled culture medium replaced. No chaser of cold thymidine will be utilized since this technique may tend to arrest further progress towards division of mammalian cells in S phase or G_1-S transition. At intervals following the pulse label, explants will be harvested, fixed and prepared for histologic sectioning.

These sections will be dipped in NTB-2 emulsion in the dark and placed in light tight boxes for 3-4 week exposures. After development they will be evaluated for thymidine index and/or labelled mitoses. For these studies it does not appear necessary to determine the true "S" time or cell cycle period, since our interest is in determining the state of viability, rather than the exact cell cycle parameters. However, the autoradiographs of the sections will be evaluated for nuclear labelling in each of the specific cell types, including: ciliated cells, mucous secretory cells, goblet cells, basal cells, intermediate cells and Clara cells when they are present.

Pulse labelling will be performed at various times during the cultivation of the tracheal or lung (bronchiole) fragments. Periods for study may include 18-24 hours, 40-48 hours, 90-96 hours, 1 week and at weekly intervals until culture is discontinued. This technique will also be utilized by pulse label technique after exposure to various carcinogens and at the specified intervals during culture indicated for the control or noncarcinogen cultures.

5. Biochemical evaluation of viability of organ cultures

Synthesis of proteins and lipids and protein secretion are acceptable indicators of energy requiring reactions in the living tissue. For determinations of protein synthesis, the fragments of trachea and lung will be incubated with uniformly labeled L-leucine-C^{14}. Incubations will be performed for 90-120 minutes at 37°C in rubber stoppered flasks containing 95% oxygen and 5% carbon dioxide in the gas phase. The reactions will be stopped by chilling, and after removal of the medium and repeated washing of the tissues, trichloracetic acid (to a final concentration of 10%) will be added. After homogenization and centrifugation, the sediment will be washed with 5% cold trichloracetic acid. Removal of lipids, phospholipids and RNA will be done according to Massaro, Weiss and Simon (36). The resulting protein precipitates will be dissolved in 1M sodium hydroxide; an aliquot will be used for protein determination by the method of Lowry et al (37) while another aliquot will be assayed for acid insoluble readioactivity. Results will be expressed in dpm/mg protein. Alternatively, incorporation of S^{35} methionine in the epithelium of trachea and lung fragments, can be examined (38).

Lipid synthesis is another parameter than can be utilized in the assessment of the viability of certain tissues. Although no information is available concerning trachea, Nasr and Heinemann (39) have demonstrated that mammalian lung tissue can incorporate acetate, palmitate and glucose into various lipids, in vitro. Fragments of lung and/or trachea will be incubated with uniformly labeled acetate C^{14} or palmitate C^{-14} for two hours at 37°C. At this time HCL will be added to a final concentration of 0.02N and the flasks shaken for 20 minutes. The tissue will be washed, homogenized and extracted by the method of Folch, Lees and Stanley (40). An aliquot will be taken for analysis of total lipid

radioactivity in a liquid scintillation counter. Results will be reported as micromoles of substrate per gram wet weight per hour.

The secretory activity of trachea and lung fragments can be measured by the isolation of isotopically labeled protein-polysaccharide complexes, such as "complex A" of Kent et al (41). Fragments of trachea or lung will be incubated at 37°C for 2-4 hours in medium containing D-glucosamine-C^{14} or sulphate -S^{35}, which are precursors of the complex. At the end of the incubation period the spent medium will be removed and applied on a Sephadex G-200 column. Aliquots will be collected for scintillation counting. Because of its high molecular weight, radioactivity incorporated into the complex is recovered in the void volume while the remaining radioactive molecules are eluted later. Results will be expressed in micromoles of substrate per gram wet weight per hour.

A normal baseline and range will be initially established for all these biochemical parameters of cell function by first examining their values in fresh tissues, immediately after death and before culture. These will be compared with those obtained at different times during organ cultures in the presence or absence of carcinogens in the various vehicles tested. The effect of vitamin A will be also studied.

6. Radioactive Isotopes for Metabolic Incorporation

Other isotopic tracers may be utilized to indicate cell viability by incorporation into metabolic products. For these studies sulfur 35 as an inorganic sodium sulfate will be utilized, and incorporation into macromolecules of acid mucopolysaccharides will be observed. Because of the energy in the sulfur isotope Beta emitter, distinct localization by audioradiography is not possible. However, similar studies utilizing tritium or carbon labeled glucosamine or L fucose can be used to study incorporation by audioradiographic techniques into the intracellular macromolecular polysaccharides. Similarly, tritiated palmitate can be used to evaluate the incorporation of phospholipid precursors into the intracellular structures of the lung. This precursor is particularly useful in evaluating synthesis of phospholipid dipalmitoryl lecithin which is believed to be synthesized in the laminated bodies of the Type II cells. Finally, the incorporation of radiolabeled carcinogenic materials will be utilized to study the incorporation, transportation and localization of these materials within the mucosal epithelial cells by audioradiography.

7. Morphometric analysis

In order to evaluate in a quantitative fashion the results of prolonged culture and effects of carcinogens, a culture tissue morphometric technique will be applied. These techniques will permit more detailed quantitative evaluation of the following parameters:

a) Number and distribution of cell types;
b) Size, including height and area of specific cell types;
c) Nuclear size and nuclear cytoplasmic ratio of specific cell types, i.e., mucous secreting cells, basal cells;
d) Number and size of mitochondria per unit of cell area;
e) Number and area of lysosomal structures per unit cell area in each specific cell type;
f) The amount of smooth and rough endoplasmic reticulum and free ribosomes per unit cell area in each specific cell type; and
g) The number, size distribution, and area of secretory granules present per unit area of secretory cell at different times during the course of the culture.

All of these analyses can be performed on the specific cell types at different times during the course of culture, and similarly at varying times after exposure to carcinogens. Morphometric analysis will be performed utilizing photographic enlargement of one micron thick Epon embedded sections of tracheal explants or electron micrographs of these structures. The techniques are those of Weibel and associates (42,43). Additional morphometric studies will be performed by image analysis methods by the application of the Quantimet 720B. This sophisticated instrument will allow us to study parameters relating to area, intercept and count from grey level analysis of histological preparations, utilizing either one micron sections or regular paraffin sections. This apparatus will provide a semi-automated method for performing these quantitating but exceedingly time consuming analyses.

8. Use of carcinogens

A variety of carcinogens have been utilized both <u>in vitro</u> and <u>in vivo</u>

in respiratory tract epithelial tissues to induce preneoplastic and neoplastic changes. In vivo studies utilizing dimethyl 1-2 benzanthracene (DMBA) in colloidal suspension (17), 3,4 benzpyrene in an aqueous Tween 60 or in olive oil (18) or sesame oil, 3-methylcholanthrene (22) have been observed to produce neoplasms by direct instillation into the tracheobronchial tree, while systemic administration of diethylnitrosamine or dimethylnitrosamine are observed to produce large numbers of epithelial tumors in the upper respiratory tract, the stomach and the larynx. Saffiotti and his associates have recently produced lower respiratory tract neoplasms by injections of 3-4 benzpyrene and hematite suspensions. Herrold (26) and others have reported the development of upper respiratory tract neoplasms after the administration of other n-nitroso compounds. In vitro studies utilizing polynuclear hydrocarbons have been reported by Lasnitzki (11), utilizing 3-4 benzpyrene by Palekar, Kuschner and Laskin (44), utilizing 3-methylcholanthrene by Dirksen, and Crocker (45) utilizing 7-12 dimethylbenz(a)anthracene (DMBA), benzpyrene, methycholanthrene and other less carcinogenic materials. To date these in vitro studies have produced abnormal states of epithelial differentiation or increased proportions of activity dividing basal cells. The ultrastructural alterations produced by DMBA and other carcinogens have been reported by Dirksen and Crocker (45). These changes consist of cytoplasm with little endoplasmic reticulum, many free ribosomes, complex autophagic vacuoles and abundant cytoplasmic filaments.

Therefore, it seems reasonable to utilize three of these carcinogenic hydrocarbons as prototypes for study of their effects on the respiratory mucosa in organ culture. These will be, a) 3,4 benzpyrene, b) 3-methylcholanthrene, c) diethylnitrosamine (DEM), and d) a relative noncarcinogen, 1,2,3,4 dibenzanthracene. 3,4 benzpyrene will be added according to the technique of Crocker (45) as an acetone solution to the growth media in a final concentration of 0.4% acetone. The actual concentrations will range from 10-15 micrograms per millimeter. Benzpyrene can also be utilized in a solution of Tween 60. The explants will be exposed continuously to this material both with and without supplementation by Vitamin A, using doses previously described.

3-methylcholanthrene will be prepared according to the techniques of Palekar, Kuschner, and Laskin (44) in a solution of Tween 60 containing 2.3 to 5.0 micrograms of methylcholanthrene per 100 milliliters of media. 1,2,3,4 dibenzanthracene (DMBA) will also be prepared according to the technique of Crocker (45) in an aqueous solution of similar concentrations containing 2.3 micrograms of DMBA per 100 milliliters of media and containing 0.1 milliliter of Tween 60.

Diethylnitrosamine (DEM) will be utilized in an aqueous solution according to the technique of Herrold (26), Montesano and Saffiotti (46) in a concentration of 1.0 to 5.0 mg per 100 ml of tissue culture media. This dose will be adjusted on the basis of the observed effects in organ culture.

These carcinogenic agents will be studied one at a time by the methods indicated. Evaluation of their effects will be performed by periodic harvest and histologic methods previously outlined. If continuous exposure to these substances produces irreversible injury, lower doses will be evaluated. In studying the effects of 3,4 benzpyrene, the effects of this material in acetone solution and in a suspension both with and without hematite and macrophages will be evaluated according to the protocol.

9. Criteria for the evaluation of malignant transformations in organ culture

Criteria for the evaluation of malignancy will be dependent on three points:

a. Histological effects of organ culture. Evidence of malignant

transformation will be evaluated on the evidence of dysplastic and truly neoplastic changes within the epithelial structures. Criteria will be epithelial metaplasia and dysplasia, loss of differentiation of the epithelial cells, pleomorphism and anaplasia of the epithelium, invasion of neoplastic cells into gland ducts and the adjacent subcutaneous tissue and exfoliation of dysplastic and neoplastic cells into the culture fluid. Epithelial change suggesting "an intraepithelial carcinoma" will also be acceptable as preliminary evidence of a neoplastic change. These changes include a more uniform but less differentiated cell type which replaces the entire epithelial surface and may extend into the glandducts.

b) The transplantability of organ culture explants will demonstrateneoplastic characteristics into inbred hamsters of the same strain. Explants which appear to have neoplastic changes will be minced into fragments no larger than 1 mm cubed and introduced subcutaneously or intramuscularly into both adult and suckling hamsters. The injected animals will be observed for growth of tumor locally and possible metastatic spread.

c) If it should grow, the transplanted tumor will be re-evaluated by histological techniques to make certain that it is the epithelial and not the mesenchymal elements which are proliferating. If growth after transplantation does not occur, the recipient animals can be prepared by cortisone pretreatment or X-Irradiation prior to transplantation in an attempt to minimize any immunologic mechanism which could be invoked against the transplanted tumor. This will be done in spite of the fact that the immune processes are not likely to be stimulated toward rejection in this inbred strain. Finally, transplantation will be attempted in an internal organ, such as the lung, which is said to be a more protected environment for growth of transplanted neoplasma than peripheral sites. If, on retransplantation, all attempts to stimulate growth are ineffective, we will attempt to dissociate the neoplastic cells and grow them in isolated culture free from mesenchymal elements. If this culture technique is successful, the epithelial component will be harvested in sufficient quantities for transplantation attempts into subcutaneous tissue and lung.